

Cryopreservation of Chlorophyll Synthesis and Apoprotein Stabilization in Barley Etioplasts¹

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Methods for the cryopreservation of protein import and integration in pea chloroplasts and of protein import or protein synthesis in tobacco mitochondria were modified to yield enzymatically active cryopreserved etioplasts from barley (*Hordeum vulgare* L.). The cryoprotectants ethylene glycol and dimethyl sulfoxide were about 64 and 77% effective, respectively, for the cryopreservation of etioplast intactness. Phototransformation of protochlorophyllide *a*, esterification of chlorophyllide *a* or zinc-pheophorbide *a*, and stabilization of the de novo synthesized plastid-encoded chlorophyll-apoproteins P700, CP47, CP43, D2, and D1 were successfully preserved in liquid nitrogen. Cryopreservation of freshly prepared intact etioplasts completely retained enzymatic activities for accumulation of chlorophyll *a* or resulted in a slightly decreased yield of zinc-pheophytin *a*.

Etioplasts isolated from 4-d-old dark-grown barley (*Hordeum vulgare* L.) seedlings are an ideal in vitro system to study the Chl-dependent accumulation of higher plant photosystems. In barley, etioplasts are formed in the absence of light from proplastids during the developmental phase of early primary leaf and plastid development (Robertson and Laetsch, 1974). Etioplasts accumulate PChlide, a Chl precursor, which is reduced to Chlide by PChlide-oxidoreductase in an NADPH-dependent reaction in the light (Apel et al., 1980). Chlide is then esterified to GGPP by Chl-synthase in a light-independent step to yield Chl (Rüdiger et al., 1980).

In the absence of Chl, etioplasts accumulated neither Chl-Ps (Herrmann et al., 1985; Klein and Mullet, 1986) nor nuclear-encoded Chl *a/b*-binding apoproteins (Apel, 1979; Bennett et al., 1984). The plastid-encoded Chl-Ps were shown to be translated and degraded at high rates (Mullet et al., 1990). During de novo synthesis of Chl from its precursors, Chlide and GGPP, Chl-Ps were shown to be stabilized against proteolytic digestion (Eichacker et al., 1990; Kim et al., 1994).

To further our understanding of the influence of Chl synthesis on the regulation of translation and Chl-P accumulation, it was necessary to prepare etioplasts by numerous time-consuming isolation steps. However, the highest activities of Chl-synthase and translation of Chl-P were obtained only when etioplasts were prepared immediately

before use. These constraints limited the number of parallel experiments that could be performed in a series. We therefore had to develop a method for the rapid and reliable cryopreservation of barley etioplasts that retained the enzymatic activities required for the stabilization of the Chl-P.

Several compounds, such as DMSO, EG, and glycerol, have been reported to act as cryoprotective agents on plant tissues (Finkle et al., 1985; Chen and Li, 1989). DMSO and EG were shown to be most effective for the cryopreservation of photosynthetic activity (Farkas and Malkin, 1979), for protein import and integration in pea chloroplasts (Yuan et al., 1991), and for protein import and in organello protein synthesis in tobacco mitochondria (Schieber et al., 1994). Here we show that the synthesis of Chl and the Chl-dependent stabilization of Chl-apoproteins can be successfully studied in cryopreserved, intact barley etioplasts.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. var Steffi) seeds were grown on moist vermiculite at 23°C in a light-tight growth chamber located in a dark room for 4 d. All manipulations of dark-grown seedlings were performed under a dim green safelight to prevent photoconversion of PChlide. ³⁵S-labeled Met was purchased from ICN-Biomedicals (Meckenheim, Germany), Percoll was from Pharmacia (Uppsala, Sweden), DMSO and EG were from Merck (Darmstadt, Germany), and glycerol was from Roth (Karlsruhe, Germany). All other chemicals were reagent grade.

Plastid Isolation and Cryopreservation

Barley seedlings (60 g) were ground in a total of 1 L of ice-cold isolation medium (0.4 M sorbitol, 2 mM EDTA, and 50 mM Hepes, pH 8.0). The slurry was sieved through a 20-μm nylon mesh and organelles were concentrated by centrifugation (3840g for 3 min) at 4°C. Etioplasts were purified by centrifugation (4097g, 10 min) on step gradients (80/40% [v/v] Percoll in 0.4 M sorbitol, 0.1% BSA, and 50 mM Hepes, pH 8.0) and intact etioplasts were collected by

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aspiration. The gradient fraction was diluted in washing medium (0.4 M sorbitol, 50 mM Hepes, pH 8.0) and intact etioplasts were concentrated by centrifugation (4097g, 2 min). Intact etioplasts were then resuspended in 400 μ L of washing buffer (50 mM Hepes/KOH, pH 8.0, 400 mM sorbitol). Intact plastids were quantitated (plastids per microliter) by counting a 500-fold dilution of the etioplast suspension in washing buffer in a hemacytometer under the phase-contrast microscope. On average, a total yield of 3×10^9 to 4×10^9 etioplasts were obtained at a concentration of 0.8×10^7 to 1×10^7 etioplasts μ L⁻¹.

For cryopreservation, 2×10^8 to 8×10^8 etioplasts corresponding to a volume of 25 to 100 μ L were transferred to 1.5-mL screw-cap vials (Sarstedt, Newton, NC). The etioplast suspension was diluted 1:2 with ice-cold DMSO, EG, and glycerol, each at a concentration of 5, 20, 40, or 60% (v/v) in 50 mM Hepes/KOH, pH 8.0, 400 mM sorbitol (DMSO* and EG*). After a 15-min equilibration on ice, etioplasts either were subjected to slow cooling rates or were directly plunged into N₂. Slow cooling rates were obtained in an insulated alcohol bath placed in a 200 K freezer. After reaching the desired temperature of about 200 K, frozen etioplasts were stored in a liquid N₂ container. For fast cooling, etioplast-containing vials were placed in a card box (NationalLab, Mölln, Germany) filled with and floating in liquid N₂. For storage, card boxes were kept in a liquid N₂ container at about 100 K (the exact storage temperature was dependent on the filling height of the N₂).

For thawing of cryopreserved etioplasts, screw-cap vials were placed in a water bath at 25°C for 30 s. The etioplast suspension was underlayered with an ice-cold cushion of 30% (v/v) Percoll in 50 mM Hepes, pH 8.0, containing 0.4 M sorbitol, and centrifuged for 3 min in an HB 4 swing-out rotor (Sorvall) at 2500g and 4°C. The upper layer containing lysed etioplasts and the Percoll cushion was removed. The pellet containing intact cryopreserved etioplasts was resuspended in 30 to 40 μ L of washing buffer to yield a final concentration of 0.5×10^7 to 1.5×10^7 etioplasts μ L⁻¹.

Gentle resuspension was achieved by tapping the bottom of the vial with a finger tip. The vial was hand-held between the thumb and first finger of the other hand and kept in a horizontal or inverted position. Cryopreserved intact plastids were quantitated as described above by phase-contrast microscopy. The percentage of cryopreserved etioplasts was determined as the ratio of the fresh isolated intact etioplasts to the total number of cryopreserved etioplasts (Percoll-cushion assay).

Unless otherwise stated, the storage time for etioplasts was between 4 and 14 d; however, no loss in enzymatic activity or intactness was found for etioplasts cryopreserved for as many as 60 d.

Chl and Protein Synthesis in Intact Cryopreserved Etioplasts

Chl and protein syntheses were performed at 25°C with etioplasts (4.2×10^7 or 1.4×10^7 plastids/assay) in a buffer mixture containing 50 mM Hepes/KOH (pH 8.0), 2.0 mM ATP/KOH (pH 7.0), 0.2 mM GTP/KOH (pH 7.0),

7 mM magnesium acetate (pH 7.0), 118 mM potassium acetate (pH 7.0), and 10 mM DTT as described by Eichacker et al. (1990), except that the translation mixture contained 400 mM sorbitol.

Protein synthesis assays were supplemented with Zn-pheide, a derivative of Chlide in which the central Mg(II) ion has been replaced by Zn(II), in a ratio of 0.3 or 1 μ M Zn-pheide to 1.3 μ M endogenous PChlide. Assays contained a 50-fold molar excess of GGPP (20 or 66.6 μ M) over Zn-pheide, which had been shown to saturate the esterification reaction catalyzed by Chl-synthase (Rüdiger et al., 1980). Reactions were incubated at 25°C for 40 min in the dark and stopped by freezing in liquid N₂ (pulse), or they were incubated for an additional 40 min in the dark, supplemented with chloramphenicol (100 μ g/mL) (chase), and then stopped. For analysis of radiolabeled proteins, etioplasts were fractionated into membrane and soluble polypeptides as described previously (Klein and Mullet, 1986). Membrane polypeptides were electrophoresed in an LKB Midget apparatus (Hoefer Scientific Instruments, San Francisco, CA) through 12.5% polyacrylamide gel in the presence or absence of 4 M urea. Gels were soaked in fluorography solution (Amplify, Amersham), dried, and exposed to x-ray film (Hyperfilm, Amersham).

Synthesis of Zn-phe or Chl in etioplasts was achieved in the dark by the addition of chemically prepared Zn-pheide or Chlide, isolated from flash-illuminated barley seedlings (Helfrich and Rüdiger, 1992) and GGPP, or by a 30-s illumination as described previously (Eichacker et al., 1990). Unless otherwise stated, assays for Chl synthesis were incubated in the dark for 80 min. Preparation and characterization of Zn-pheide and Chlide and the determination of Zn-phe or Chl esterified during *in vitro* reactions were carried out as described by Helfrich et al. (1994). Percentage of esterification was determined as the ratio of Zn-phe or Chl synthesized at 25°C and Zn-pheide or Chlide isolated from parallel assays kept on ice.

Reactions were carried out in triplicate. Results from at least two independent cryopreservation reactions were used to calculate the mean values given. Error bars represent SD.

Electron Microscopy Analysis

Etioplasts were fixed overnight in 2.5% glutaraldehyde, 400 mM sorbitol, 2 mM MgCl₂, 75 mM dimethylarsinic acid sodium salt-trihydrate/HCl (cacodylate buffer, pH 7.0). Etioplasts were pelleted and washed sequentially three times in washing buffer for 15, 25, and 15 min and in distilled water for 15, 15, and 60 min and then kept in water overnight. Samples were postfixed in 1% OsO₄ and then dehydrated in a graded acetone series from 10, 20, 40, 60, 80, to 100%, in which staining with 1% uranyl-acetate was achieved during dehydration in 20% acetone. Dehydrated etioplasts were embedded in Spurr's resin and polymerized at 65°C overnight. Ultrathin sections were poststained with lead citrate for 1 min. Sections were examined under an electron microscope (model EM 912, Zeiss).

RESULTS AND DISCUSSION

Cryopreservation of Etioplast Intactness

Several agents and procedures that successfully cryopreserve the structure and selected functions of plant organelles and subfractions have now been described (Finkle et al., 1985; Chen and Li, 1989). Although there are still no absolute rules for cryopreservation, agents such as EG or DMSO, in combination with fast cooling rates, have been reported to be superior to glycerol and slow cooling rates (Farkas and Malkin, 1979; Yuan et al., 1991; Schieber et al., 1994).

To establish a protocol for the cryopreservation of intact etioplasts from barley, we tested the freezing of etioplasts at slow (about 1°C/min) and fast (directly in liquid N₂) cooling rates after an 15-min equilibration on ice in the presence of DMSO, EG, or glycerol. The number of etioplasts that remained intact upon thawing at 25°C was determined on the basis of Percoll-cushion assays (see "Materials and Methods"). When 8×10^8 etioplasts were subjected to slow rates of cooling in a volume of 200 μ L and a 10% solution of either DMSO* or EG*, yields of cryopreserved etioplasts were highly variable, and mean deviations of more than 40% were observed between experiments (data not shown).

When the intactness of etioplasts was investigated under identical conditions using fast cooling rates, more consistent yields were achieved, and mean deviations were less than 20% of the yields measured in separate experiments. Mean values of 77 and 65%, respectively, were obtained for the cryopreservation of intact etioplasts in the presence of the cryoprotectant DMSO* or EG* (Fig. 1). When etioplasts were cryopreserved in a 10% (v/v) solution of glycerol in 50 mM Hepes/KOH, pH 8.0, and 400 mM sorbitol, an

average of only 40% of the intact etioplasts were protected from lysis, and the mean deviation of the intact etioplasts obtained was 30% in three experiments from separate etioplast preparations (Fig. 1). Upon increasing the cryoprotectant concentration from 2.5 to 30% (v/v), an optimum was found for the cryopreservation of etioplasts at solutions of 10% (v/v) DMSO, at 10 to 20% (v/v) of EG, or between 2.5 and 20% (v/v) glycerol in 50 mM Hepes/KOH, pH 8.0, and 400 mM sorbitol (Fig. 1). We further tested Suc concentrations between 20 and 50% (w/v) in washing buffer for a cryopreserving influence on etioplast intactness and found that these concentrations had no effect.

Optimum cryopreservation of barley etioplasts and pea chloroplasts required the same concentration of EG, whereas a lower concentration of DMSO was sufficient to cryoprotect barley etioplasts (Yuan et al., 1991). Although cryopreservation of pea chloroplasts and barley etioplasts was poor in the presence of glycerol, twice the yield of etioplasts could be obtained in 20% glycerol. It is interesting to note that lysis of barley etioplasts and pea chloroplasts occurred at a concentration of 30% (v/v) EG, whereas mitochondria remained intact at high yields (Schieber et al., 1994). These data could indicate in general that the envelope membranes of plant mitochondria are less sensitive to internal ice crystal formation and osmotic imbalances during freezing than the envelope membranes of etioplasts and chloroplasts. This is also indicated by the finding that, upon freezing in the absence of cryoprotectants, neither barley etioplasts nor pea chloroplasts could be re-isolated intact, whereas about 50% of the tobacco mitochondria retained their intactness (Yuan et al., 1991; Schieber et al., 1994). Because of the poor cryopreservation of barley etioplasts in glycerol or Suc, only the plastids preserved in DMSO* or EG* were utilized in subsequent experiments.

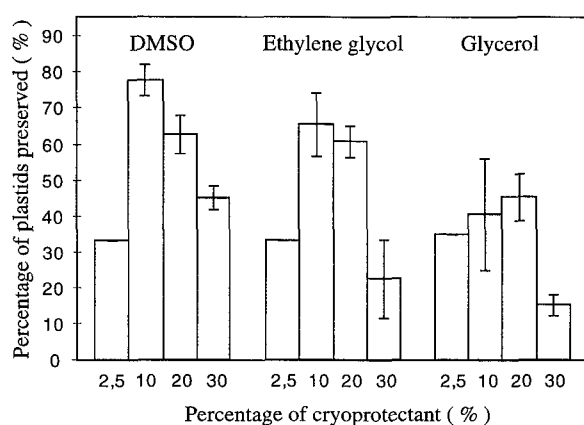


Figure 1. Cryopreservation of etioplast intactness. Etioplasts were isolated and dissolved in 2.5, 10, 20, and 30% (v/v) of the cryoprotectants DMSO, EG, or glycerol, as described in "Materials and Methods," equilibrated for 15 min on ice, and frozen in liquid N₂. They were then thawed at room temperature and intact plastids were re-isolated on Percoll gradients. Etioplasts were counted in a hemacytometer and the percentage of intact plastids was calculated as the ratio of the number of intact plastids determined after and before freezing. Values are the means of at least two independent cryopreservation reactions containing 2.5×10^8 plastids. Bars represent SD.

Microscopic Examination of Etioplast Integrity and Ultrastructure

Intactness of the etioplasts was verified by phase-contrast microscopy, and ultrastructure was examined by thin-section electron microscopy, neither of which revealed any structural differences between etioplasts isolated fresh and those cryopreserved in DMSO* or EG* and fixed in 2.5% (v/v) glutaraldehyde (Fig. 2). Intact, cryopreserved etioplasts showed the key structural elements of intact etioplasts: an envelope membrane system bordering a highly granular stroma fraction containing the inner prolamellar body and prothylakoid membrane system and numerous plastoglobuli bodies. Cryopreserved etioplasts ranged in size from 1.5 to 3.0 μ m and showed no cryoprotectant-dependent swelling or shrinking at the optimum cryoprotectant concentrations (Fig. 1).

Cryopreservation of Chl Synthase Activity

To test whether DMSO* or EG* alone could have an inhibitory effect on the capability of etioplasts to synthesize Chl, fresh isolated plastids were preincubated on ice either

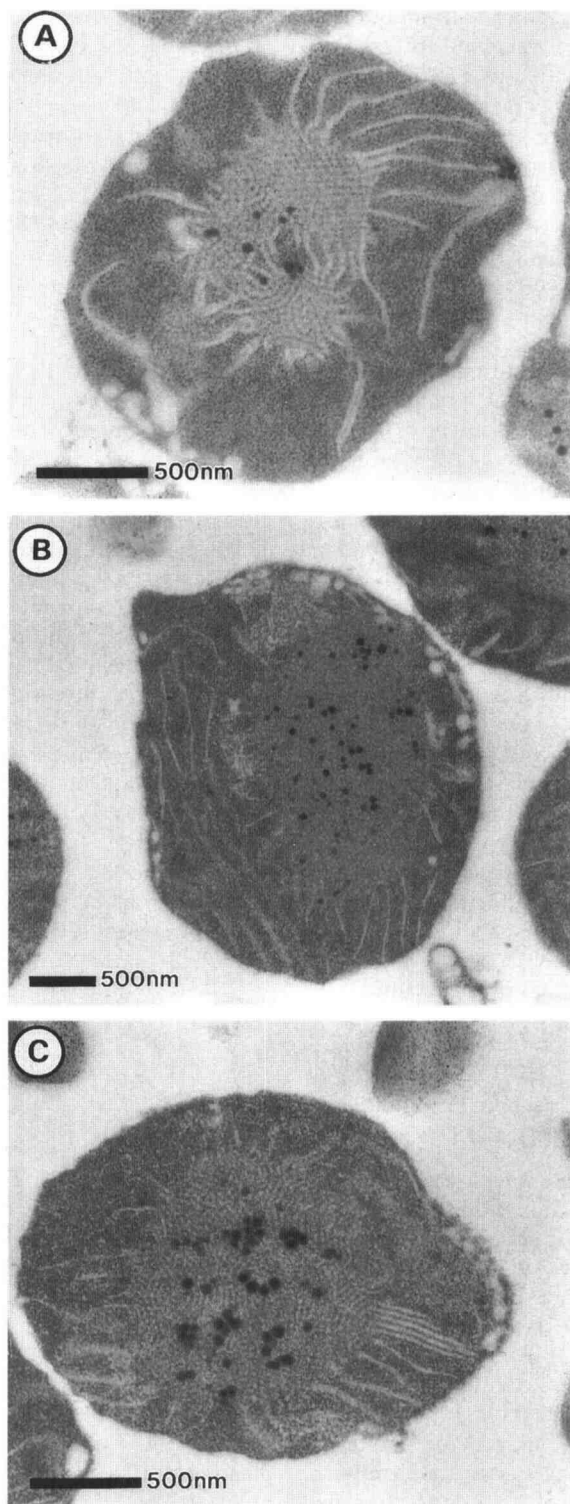


Figure 2. Ultrastructure of fresh and cryopreserved etioplasts. Etioplasts were prepared fresh (A) or cryopreserved in DMSO* (B) or EG* (C) and fixed in a buffered, iso-osmotic glutaraldehyde solution. Ultrathin sections were analyzed. Magnifications of electron micrographs are given in size bars (nm).

in washing buffer or in DMSO* or EG* for 15 min (Fig. 3, columns 1 and 4; 2 and 5; and 3 and 6). Then etioplasts were washed, re-isolated, and assayed for Chl-synthesis activity. Zn-pheide, defined previously, was used to monitor selectively the effect of cryoprotectants on the esterification reaction. Phototransformation of endogenous PChlide was used to investigate the sensitivity of the PChlide-oxidoreductase to the freezing of etioplasts in the presence of cryoprotectants. Etioplasts preincubated in washing buffer, DMSO*, or EG* esterified an average of 67, 64, and 77%, respectively, of the Chlide and 49, 45, and 40%, respectively, of the Zn-pheide added to the reactions (Fig. 3, columns 1–3 and 4–6). Zn-pheide was esterified best in freshly prepared etioplasts (Fig. 3, column 4), whereas preincubation in DMSO* and EG* decreased the capacity of etioplasts to accumulate Zn-phe (Fig. 3, columns 5 and 6). The synthesis of Chl from endogenous Chlide was increased over that obtained in washing buffer after preincubation of etioplasts in EG* (Fig. 3, column 1 versus 3), indicating that EG* influenced the capacity of the etioplasts to synthesize Chl from endogenous Chlide during preincubation. Thus, preincubation of etioplasts in EG* is superior to preincubation in washing buffer, yielding the highest levels of Chl formation (Fig. 3, column 3).

We next tested the Chl-synthesizing activity of etioplasts after freezing the plastids in the cryoprotectants (Fig. 3,

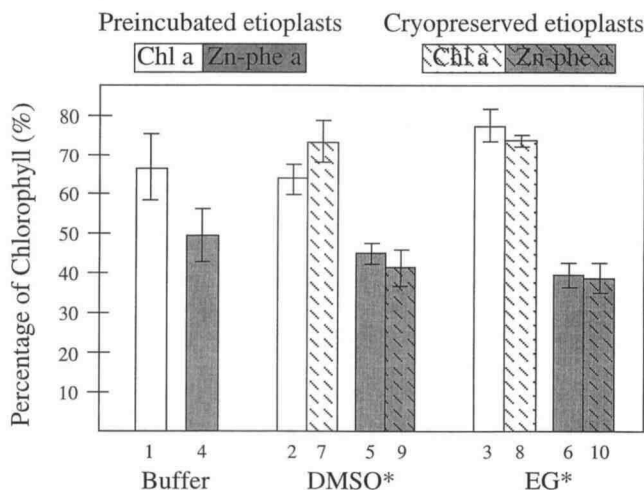


Figure 3. Influence of cryoprotectants on the synthesis of Chl or Zn-phe. Intact etioplasts were tested for their competence at synthesizing Chl after preincubation (columns 1–6) or after freezing (columns 7–10). Etioplasts were incubated with DMSO* (columns 2, 7, 5, and 9), EG* (columns 3, 8, 6, and 10), or a standard reaction buffer (columns 1 and 4) for 15 min on ice. Intact plastids received Chlide through the photoreduction of endogenous PChlide or Zn-pheide through exogenous addition. The percentage of Chl or Zn-phe synthesized was determined as the ratio of the amount of Chl synthesized to Chlide formed or as the ratio of the amount of Zn-phe synthesized to Zn-pheide added to the etioplast suspension. Assays contained 4.2×10^7 plastids, corresponding to 270 pmol (preincubated etioplasts) or 315 pmol (cryopreserved etioplasts) of endogenous PChlide, and received 675 pmol of Zn-pheide (preincubated etioplasts) or 1 nmol of exogenously added Zn-pheide (cryopreserved etioplasts). Values are the means of at least two independent cryopreservation reactions. Bars represent SD.

columns 7–10). Etioplasts frozen in DMSO* or EG* revealed no significant difference in their capacity to synthesize Chl (Fig. 3, columns 7 and 8). However, freezing plastids in DMSO* was superior to preincubating them in DMSO* (Fig. 3, columns 2 and 7). Also, etioplasts frozen in DMSO* or EG* retained higher Chl yields than those preincubated in washing buffer (Fig. 3, column 1 versus 7 and 8), indicating that freezing plastids in cryoprotectants alters their capacity for Chl synthesis. For example, the yield of Zn-phe was about 40% of the Zn-pheide added exogenously to the etioplasts cryopreserved in DMSO* and EG* (Fig. 3, columns 9 and 10). Synthesis of Zn-phe was therefore about 30 to 50% less efficient than synthesis of Chl from the natural precursor Chlide, endogenously formed within the intact etioplasts after photoreduction.

When the esterification reaction in intact etioplasts was compared with that in etioplasts broken by lysis through omission of sorbitol in the reaction assays or by freezing and thawing in the absence of a cryoprotectant, both methods decreased the accumulation of Zn-phe (by a factor of 1.3 and 1.7, respectively [Fig. 4, Zn-phe a, lysed, frozen]). Freezing etioplasts in the absence of a cryoprotectant decreased the accumulation of Chl about 3-fold in comparison with that in etioplasts lysed by the absence of sorbitol (Fig. 4, Chl a, lysed, frozen). This suggests that freezing affects the phototransformation reaction more than it affects the esterification reaction. Freezing in the presence of a cryoprotectant could thus increase the yield of Chl through stabilization of the phototransformation of endogenous PChlide (Fig. 3, column 7 or 8 versus 1).

Cryopreservation of Chl Apoprotein Accumulation

The *in vitro* accumulation of Chl-P in the etioplast inner membrane system requires etioplasts capable of (a) trans-

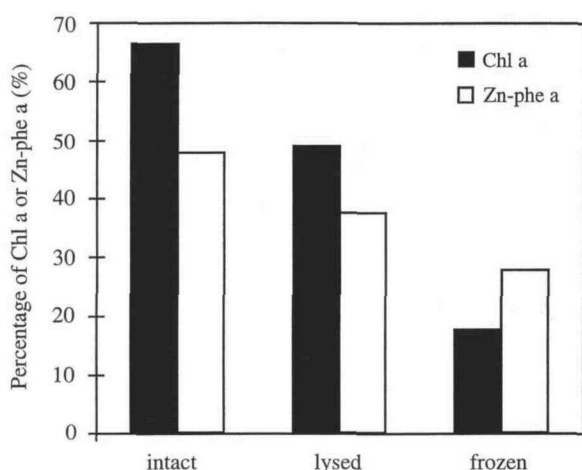


Figure 4. Influence of etioplast intactness on accumulation of Chl or Zn-phe. Etioplasts (400 pmol PChlide) were prepared fresh and tested for their capacity to accumulate Chl or Zn-phe. During the reaction, etioplasts were kept intact, lysed in a reaction medium without sorbitol, or added to the reaction after lysis of the etioplasts through a freeze/thaw treatment. Etioplasts received a mean of 400 pmol of Zn-pheide or 300 pmol of endogenous Chlide and a 12.5-fold molar excess of GGPP for esterification. Reactions were run for 80 min.

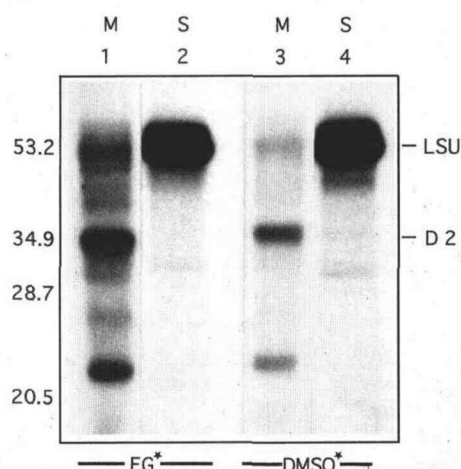


Figure 5. Protein synthesis in cryopreserved etioplasts. Intact etioplasts (1.4×10^7 plastids) were cryopreserved in DMSO* or EG* as described in "Materials and Methods." Re-isolated etioplasts were used for a 40-min pulse labeling of proteins with [35 S]Met. Thereafter, etioplasts were lysed in 50 mM Hepes-KOH, pH 8.0, 10 mM magnesium chloride, 25 mM potassium acetate and fractionated into a membrane phase (M, lanes 1 and 3) and a soluble phase (S, lanes 2 and 4). A phase volume equivalent of 4×10^5 plastids was loaded per lane on a 12.5% polyacrylamide gel containing 4 M urea. The gel was fixed, fluorographed, and exposed to x-ray film for 64 h. Migration of molecular weight markers is given to the left of the figure, and proteins identified with the membrane or soluble phase are labeled D2 and LSU, respectively.

lating endogenous membrane-bound mRNAs, (b) binding of cofactors and of the *de novo* synthesized pigments to *de novo* synthesized Chl-P, and (c) folding and membrane integration of the apoproteins. Etioplasts contain membrane-bound and mRNA-associated polysomes for all Chl-Ps. Polysomes can be read out *in vitro* to yield full-length apoproteins associated with the membrane phase, although they show a considerable degree of pausing during run off (Kim et al., 1991, 1994).

To test the parameters responsible for stable accumulation of *de novo* synthesized Chl-P, we first compared the capability of etioplasts cryopreserved in DMSO* or EG* to translate endogenous mRNAs and accumulate membrane-bound and soluble polypeptides (Fig. 5). Incorporation of radiolabel during the translation of proteins associated with the soluble phase was mainly into the LSU and was about equal under both conditions (Fig. 5, lanes 2 and 4; LSU). However, the apoprotein pattern associated with the membrane phase (Fig. 5, lanes 1 and 3; D2) showed a higher incorporation of radiolabel into full-length apoproteins if etioplasts were cryopreserved in EG*. Also, the background labeling between apoprotein bands was considerably higher in the presence of EG* as a cryoprotectant, indicating either (a) a less efficient read out of mRNA and accumulation of translation intermediates or (b) a less-efficient degradation of translation intermediates and full-length Chl-P, resulting in the accumulation of radiolabeled Chl-P and translation intermediates (Fig. 5, lane 1 versus 3).

Next we compared the capability of the cryopreserved *in vitro* system to translate and stabilize Chl-P against pro-

teolytic digestion in etioplasts isolated fresh and in those cryopreserved in DMSO* during pulse and pulse/chase treatments (Fig. 6). Apoproteins were pulse-labeled in intact etioplasts in the absence and presence of Chl synthesis and the SDS-PAGE pattern of radiolabeled apoproteins was compared (Fig. 6, lanes 1–3). The identities of cryopreserved etioplast translation products were verified by immunoprecipitation with antibodies directed against CP47, CP43, and D1 (data not shown).

The stabilization of apoproteins against proteolytic digestion was investigated by pulse labeling, followed by further incubation of etioplasts with chloramphenicol, which arrests plastid ribosomes. This ensured that no new

radiolabel became incorporated into full-length apoproteins during the proteolytic breakdown of apoproteins not assembled with Chl (Fig. 6, lanes 4–6). The selective pigment requirements of Chl-Ps for stabilization by Zn-phe required the use of two different ratios of Zn-pheide to PChlide (Fig. 6, lanes 2 and 3 and 5 and 6).

In the absence of Zn-phe synthesis, neither fresh nor cryopreserved etioplasts accumulated P700, CP47, or CP43 in the membrane fraction, whereas the D1 and D2 protein accumulated during the pulse-labeling (Fig. 6, lanes 1). However, only D2, which is known to require no Chl synthesis for accumulation (Eichacker et al., 1990), and an aliquot of the LSU remained bound to the membranes and resistant to proteolysis during the period of chloramphenicol incubation, whereas D1 was degraded (Fig. 6, lanes 4 versus 1).

Upon esterification of Zn-pheide, all Chl-Ps accumulated and were also stabilized against degradation in freshly isolated as well as cryopreserved etioplasts (Fig. 6, lanes 2 and 3 and 5 and 6). However, stabilization of the Chl-Ps P700, CP43, and D1 through the synthesis of Zn-phe was not as effective in cryopreserved etioplasts (Fig. 6B, lanes 3 versus 6) as it was in freshly prepared etioplasts (Fig. 6A, lanes 3 versus 6). This effect was most pronounced for low Zn-phe concentrations, whereas higher amounts of Zn-phe yielded higher stabilization of Chl-P within the cryopreserved etioplasts (Fig. 6B, lanes 2 versus 5).

These results indicate that cryopreserved intact etioplasts can be successfully used to study the synthesis of Chl or Zn-phe and the translation stabilization of the de novo synthesized Chl-Ps. The system described will now be used to study the assembly of stabilized Chl-Ps.

CONCLUSIONS

The data presented here may be of special use in studies of the regulation of the biogenesis of the higher plant photosystems I and II. The cryopreservation method consisted of the following steps: (a) flash freezing of 200 μL of 1×10^6 to 4×10^6 etioplasts μL^{-1} in liquid N_2 in the presence of either DMSO* or EG*; (b) thawing at 25°C for 30 s; (c) purification of cryopreserved etioplasts at 2500g on 30% Percoll in 50 mM Hepes/KOH, pH 8.0, 400 mM sorbitol for 3 min; and (d) resuspension of the intact etioplasts in 30 to 40 μL of washing buffer to obtain a final concentration of about 2×10^7 etioplasts μL^{-1} . The method is fast, simple, and reproducible for the cryopreservation of intact etioplasts. Therefore, the time-consuming process of the fresh preparation of etioplasts can now be done ahead of time, not necessarily on the day of the experiment.

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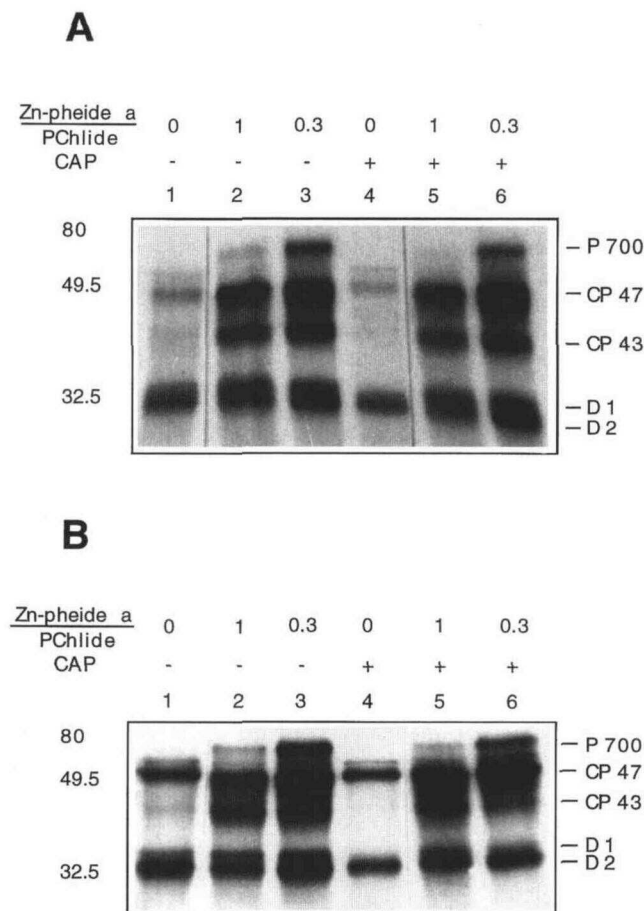


Figure 6. Cryopreservation of Chl-P accumulation. Accumulation of Chl-P was assayed in intact etioplasts isolated fresh (A) or cryopreserved for 7 d (B). Intact etioplasts were pulse-labeled for 40 min (lanes 1–3) or were subjected to pulse labeling for 40 min and treated with chloramphenicol (CAP) for an additional 40 min (lanes 4–6). Assays were performed in the absence (lanes 1 and 4) or in the presence of Zn-phe synthesis (lanes 2 and 3 or 5 and 6). Zn-pheide was added to the reactions in a molar ratio to the endogenous PChlide of either 1 (lanes 2 and 5) or 0.3 (lanes 3 and 6). Etioplasts were fractionated into a membrane phase and a soluble phase, and a membrane fraction equivalent of 4×10^5 etioplasts was separated on a 12.5% SDS-PAGE gel. The gel was fixed, fluorographed, and exposed to x-ray film for 40 h. Migration of molecular weight markers is given to the left of the figure and proteins identified with the membrane phase are labeled P700, CP47, CP43, D1, and D2.

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